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Bioactive Constituents, Metabolites, and Functions

**Cocoa flavanols improve endothelial functional integrity in healthy young and elderly subjects**

Michael Gröne, Roberto Sansone, Phillip Höffken, Patrick Horn, Ana Rodriguez-Mateos, Hagen Schroeter, Malte Kelm, and Christian Heiss

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1 **Cocoa flavanols improve endothelial functional integrity in healthy young and elderly subjects**

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**ABSTRACT**

Cocoa flavanols (CF) can improve flow-mediated dilation (FMD), blood pressure, and vascular stiffness in healthy subjects. Endothelial microparticles (EMPs) are markers of endothelial functional integrity reflecting activation and injury. In plasma samples, we investigated whether age-dependent changes in circulating EMPs exist and whether CFs decrease EMPs in healthy humans. The concentrations of CD31<sup>+</sup>/41<sup>-</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup>-EMPs (flow-cytometry) were increased in healthy elderly (n=19) as compared to young (n=20) non-smokers. EMPs correlated with age, systolic blood pressure, and pulse wave velocity. CD31<sup>+</sup>/41<sup>-</sup> and CD62e<sup>+</sup>-EMPs inversely correlated with FMD. Following 2 weeks twice-daily CF consumption (450 mg), CD31<sup>+</sup>/41<sup>-</sup> and CD144<sup>+</sup>-EMPs decreased both in young and elderly subjects compared to CF-free control. The EMP decrease inversely correlated with FMD improvements. Cardiovascular aging is associated with increased EMPs that can be modulated by dietary flavanols along with improvements in vascular function. This indicates that flavanol consumption can improve endothelial functional integrity in healthy humans.

**Key words:** age; endothelial microparticles; endothelial function; nutrition; blood pressure; arterial stiffness

## INTRODUCTION

Vascular aging is a complex continuous multifactorial process leading to gradual loss of vascular function and development of structural changes including arterial stiffness and loss of microvasculature. These changes promote the development of many age-related diseases and account for a large proportion of cardiovascular mortality.<sup>1</sup> The mechanisms underlying vascular aging are not fully understood but involve all segments of the arterial vascular system and overlap with those of atherosclerosis and arteriosclerosis. An impairment in endothelial homeostasis may be a common central mechanism.<sup>2</sup>

More recently, interventions to slow cardiovascular aging have become an important focus of research aiming at prevention of cardiovascular disease.<sup>2</sup> In this context, diet and specific plant bioactives may be of interest.<sup>3</sup> In particular flavanols, a subgroup of dietary plant-derived bioactives that is mostly consumed with tea, pome fruits, berries, and cocoa products,<sup>4</sup> have received much attention, as clinical studies indicate that a higher intake of flavanol-containing foods can not only improve arterial function in individuals at risk for cardiovascular disease (CVD) and with established CVD<sup>5, 6</sup> but also healthy individuals over a broad age range.<sup>7, 8</sup> These clinical intervention trials have shown that flavanols can improve cardiovascular parameters implied in vascular healthy aging including endothelial function, arterial stiffness, blood pressure, cholesterol, and potentially glycemic control.<sup>3, 9</sup> For instance in the FLAVIOLA Health study, we have studied the effect of a dietary cocoa flavanol (CF) intervention on hallmarks of cardiovascular aging on major segments of the cardiovascular system, such as cardiac performance, endothelial dysfunction, increased systolic blood pressure, vascular stiffness, and microcirculatory functions in healthy humans.<sup>7, 8</sup> The results showed that a 2-4 week CF intervention can reverse age-related burden of cardiovascular risk in healthy elderly and middle aged subjects with significant improvements in flow-mediated dilation and microvascular perfusion, decrease in systolic blood pressure, pulse wave velocity, and aortic augmentation index.<sup>7, 8</sup> Whether these changes led to improved endothelial functional integrity in elderly humans is unknown so far.

To further investigate the role of functional endothelial integrity in age-dependent vascular responses to CF, we measured circulating endothelial microparticles (EMPs), which are endothelium

45 specific biomarkers of functional integrity reflecting endothelial activation and injury,<sup>10-15</sup> in samples  
46 from our previous study.<sup>7</sup> Specifically, in the current analysis, we investigated (a) whether age-  
47 dependent changes in circulating EMPs exist, (b) whether these correlate with parameters of vascular  
48 function and structure that change with age, and (c) whether the CF intervention also affected  
49 circulating EMPs along with improved vascular function in healthy humans.

## **MATERIALS AND METHODS**

### **Study participants and study design**

To determine the impact of age on circulating endothelial microparticles, we analyzed plasma samples from 20 young (<35 years) and 19 elderly (50-80 years) healthy male Caucasian adult subjects that were obtained during our previous study (Table 1).<sup>16</sup> We then analyzed the impact of a 2-week cocoa flavanol intervention on plasma EMP concentrations. As previously published,<sup>16</sup> both young and elderly participants were randomly assigned to either the CF intake group (FLAVANOL; 450 mg total flavanols twice daily) or a nutrients-matched CF-free group (CONTROL) based on a double-masked, parallel group study design (See supplemental table 1 for characteristics of individual groups). Plasma samples were taken after overnight fasting on day 1 and day 14.

The results and detailed methods of hemodynamic endpoints were previously published.<sup>16</sup> Briefly, central arteries, especially the aorta, were characterized by their physicommechanical properties estimating central blood pressure (BP), pulse wave velocity (PWV), and augmentation index (AIX) with the Sphygmocor device (AtCor Medical); office blood pressure was measured on the upper arm using a standard sphygmomanometric cuff (Dynamap), conduit artery function was measured by ultrasound (Vivivi, GE) as brachial artery flow-mediated dilation (FMD) following 5 min occlusion of the forearm with a blood pressure cuff (Brachial Analyzer) and nitroglycerin-mediated vasodilation (NMD) at 3 min after 400 mg nitroglycerin SL (Nitrolingual, Pohl). Maximal perfusion of the cutaneous microcirculation was assessed by Laser Doppler perfusion imaging (Perimed) after 5 min of lower arm occlusion. The study protocol was approved by the ethics committee of the Heinrich-Heine-University Duesseldorf; all subjects gave written informed consent. (Clinicaltrials.gov: NCT01639781).

### **Test materials**

As previously described in detail,<sup>16</sup> both interventions provided by Mars, Inc. used a low-calorie fruit-flavored beverage mix that was standardized and matched in composition. The CF-containing drink (FLAVANOL) provided 450 mg of total cocoa flavanols per serving.<sup>17</sup> The total amount of CF in mg represents the sum of all monomeric flavanols and their oligomers (i.e., procyanidins) with a degree of

polymerization up to and including 10 (i.e. DP 1-10). (-)-Epicatechin was the predominant monomeric flavanol in this drink. Compositional details for the CONTROL and FLAVANOL test drinks are provided in Table 2.

### **Characterization of EMP subpopulations by flow cytometry**

Six mL of cubital venous blood was drawn into citrate containing Vacutainer tubes and processed within 2 h. Platelet-rich plasma (PRP) was acquired by centrifugation of whole blood at 300 g over 15 min at room temperature (RT). Platelet-free plasma (PFP) was obtained by 2 successive centrifugations of PRP at 10,000 g for 5 min at RT. As described previously, microparticle subpopulations were discriminated by flow cytometry according to the expression of established surface antigens.<sup>18</sup> PFP was incubated for 30 min with fluorochrome-labeled antibodies or matching isotype controls and analyzed in a flow cytometer (VERSE, Beckton Dickinson, Heidelberg, Germany). Microbead standards (1.0  $\mu\text{m}$ ) were used to define an analysis gate consistent with the size of EMPs. Events of less than 1.0  $\mu\text{m}$  diameter were identified in forward scatter and side scatter intensity dot representation, gated as microparticles, and then plotted on 1- or 2-color fluorescence histograms. The MP subpopulations were defined as elements that were less than 1.0  $\mu\text{m}$  in size and that were positively labeled by the specific antibodies for the endothelial surface markers CD31<sup>+</sup>/CD41<sup>-</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup>. The concentration of MPs was quantified by comparison of flow-count calibrator beads (20  $\mu\text{L}$ ) with a predetermined concentration and expressed as events per microliter PFP (Ev/ $\mu\text{L}$ ).

### **Statistical methods**

Demographics are given as mean and standard deviation. Results as mean and standard error of the mean. Young and elderly plasma EMP concentrations were compared using independent student t-test. Changes in EMP concentration (Delta) were calculated as 2-week concentration minus baseline concentrations for each individual. The primary test for an effect of CF was a two-way ANOVA (2 between subject factors: intervention (CONTROL/FLAVANOL) and age (young/elderly). ANOVA



106 was performed with SPSS (V 24; IBM). Univariate correlations were Pearson's  $r$ . P values of less than  
107 0.05 were regarded as statistically significant.

## RESULTS

Baseline demographic, clinical, and hemodynamic characteristics of young ( $26 \pm 3$  years) and elderly ( $60 \pm 8$  years,  $p < 0.001$ ) study subjects are presented in Table 1 and Supplemental Table 1 baseline characteristics split by treatment group). Note that values slightly differ from our previous publication,<sup>16</sup> as only values of subjects with EMP data were included (young  $n=20$ ; elderly  $n=19$ ). All subjects were non-smokers, were non-diabetic, had no diagnosis, present or history of clinical symptoms of cardiovascular disease (dyspnea, angina, edema, claudication, palpitations). None were not taking regular medication or had a medical indication for blood pressure or cholesterol lowering medication. The elderly study group exhibited statistically significant greater body weight, fasting glucose, total and LDL cholesterol. Furthermore, the elderly had a higher diameter of brachial artery, office and central systolic blood pressure, central diastolic blood pressure, PWV, AIX, and lower FMD and maximal microvascular perfusion.

As depicted in Table 3, age correlated with fasting glucose, BMI, total cholesterol and LDL cholesterol. Positive correlations existed between age and brachial artery diameter, office and central blood pressure, PWV, and AIX. Inverse correlations existed between age and FMD and maximal microvascular perfusion.

### Age-related increase in circulating concentrations of endothelial microparticles

Young subjects exhibited significantly lower concentrations of CD31<sup>+</sup>/41<sup>-</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup> EMPs in plasma than elderly subjects (Figure 1). EMPs and age showed significant inverse correlations (Table 3). We observed statistically significant correlations of between EMP concentrations and hemodynamic parameters that go along with increased mechanical stress of large arteries including systolic blood pressure, PWV, and AIX.

### Flavanol intervention decreases EMPs in healthy young and elderly humans and is associated with improvements in endothelial function

FLAVANOL intake led to a significant decrease of CD31<sup>+</sup>/41<sup>-</sup> (main effect  $p=0.003$ ), CD144<sup>+</sup> ( $p=0.001$ ), and CD62e<sup>+</sup>-EMPs ( $p=0.006$ ) in both young and elderly subjects as compared to

CONTROL (Figure 2). The interaction between age and intervention were not statistically significant suggesting that the effect size did not differ with age ( $CD31^{+}/41^{-}$ :  $p=0.691$ ;  $CD144^{+}$ :  $p=0.813$ ;  $CD62^{+}$ :  $p=0.489$ ). As previously reported, FLAVANOL led to a statistically significant increase in FMD and microvascular perfusion and lowering in cholesterol, blood pressure, and PWV both in young and elderly healthy subjects.<sup>19</sup> AIX was only significantly decreased in elderly.<sup>19</sup> We observed significant inverse correlations between changes in FMD and EMPs (Table 4). Furthermore, the changes in  $CD31^{+}/41^{-}$  and  $CD62e^{+}$ -EMPs correlated with the changes in central systolic blood pressure. The changes in PWV correlated with the changes in  $CD144^{+}$  and  $CD62e^{+}$ -EMPs.

## DISCUSSION

Our data demonstrate that at baseline the levels of circulating endothelial microparticles were higher with age and correlated with hemodynamic parameters associated with ageing. Furthermore, our data show that a 2-week cocoa flavanol intervention significantly decreased EMPs in both healthy young and elderly volunteers and the changes in EMPs correlated with improvements of hemodynamic parameters.

In the presence of cardiovascular risk factors, concerted with a genetic disposition and environmental factors, the arterial endothelium loses its normal regulatory function for vessel wall homeostasis; a concept termed ‘endothelial dysfunction’.<sup>20</sup> Cardiovascular risk factors appear to converge both in positive, but also in negative ways, on the vascular endothelium with significant effects on the progression vascular aging.<sup>21</sup> Flow-mediated dilation is an established methodology to measure endothelium-dependent vasodilation as a surrogate marker for cardiovascular risk. However, FMD measures the capacity of an artery to dilate in response to stimulus and this can be affected by non-endothelial factors such as the response of smooth muscle cells or structural components of the arterial wall. EMPs can be seen as circulating biomarkers of a compromised endothelial integrity that are released from activated and apoptotic endothelial cells.<sup>13, 14</sup> Circulating levels of EMPs increase in plasma early in atherosclerotic processes, correlate with the degree of endothelial dysfunction,<sup>22</sup> and have been established as prognostic biomarkers that predict adverse CV outcome.<sup>23-25</sup> Cardiovascular risk factors including hypertension, hypercholesterolemia, and smoking were shown to trigger EMP release.<sup>26, 27</sup> The current study is the first to demonstrate that EMPs are age-dependently increased in healthy humans and that EMPs correlate with changes in hemodynamic parameters that occur during healthy aging. We propose that, this increase may be due to endothelial activation (CD62e<sup>+</sup>), ongoing mechanical endothelial injury (CD144<sup>+</sup>), or impaired vascular protection due to lowered wall shear stress (CD31<sup>+</sup>/41<sup>-</sup>) similar to arterial hypertension.<sup>10</sup>

A number of clinical intervention studies have shown that the intake of flavanol-containing foods or isolated flavanols can improve cardiovascular risk biomarkers including FMD and blood pressure in individuals at risk for CVD, with established CVD<sup>5, 6</sup>, and healthy individuals at low CVD risk over a broad age range.<sup>7, 8</sup> Although the mechanisms of action underlying the biological effects of

flavanols are not completely understood, flavanols are one of a few bioactives known today, for which causality between the intake and an improvement in FMD has been demonstrated.<sup>28</sup> As FMD reflects endothelium-dependent vasodilation,<sup>29</sup> improvements in FMD are paralleled by increased plasma concentrations of nitric oxide species,<sup>6, 30</sup> and FMD improvements were inhibited by intravenous application of an nitric oxide synthase inhibitor<sup>30</sup> it is currently believed that cardiovascular effects of flavanols may be due to effects on the vascular endothelial cells.<sup>29</sup> Only a few studies are available on the effect of bioactives on EMPs in humans.<sup>31-33</sup> We have previously shown in patients with coronary artery disease that CF related improvements in FMD and circulating nitric oxide species were paralleled by significant decreases in circulating EMPs supporting the concept that flavanols may improve endothelial integrity in patients with CVD.<sup>33</sup> In the current manuscript, we extend these findings to healthy young and elderly humans supporting that CF specifically increase functional endothelial integrity not only in patients with coronary artery disease,<sup>33</sup> but also healthy people. Furthermore, as CD62e<sup>+</sup> is exclusively expressed on the surface of activated endothelial cells, a decrease in CD62e<sup>+</sup> EMPs as seen in our present work can be cautiously interpreted as a decrease in endothelial inflammation or activation even in ‘healthy’ humans.<sup>32</sup> Others have shown *in vitro*,<sup>34</sup> that nitric oxide can blunt EMP release via ABCA1 expression and cytoskeletal reorganization offering a potential mechanism of how CF may decrease EMPs; a hypothesis that awaits *in vivo*.

Taken together, we conclude that healthy human cardiovascular aging is associated with increased endothelial microparticles along with decreased vascular function that can be modulated by a dietary flavanol intervention along with improvements in vascular function. Our data further support that an intervention with dietary CF improves endothelial functional integrity in healthy humans.

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**Table 1:** Baseline (A) clinical and (B) hemodynamic characteristics of study population (mean [SD], t-test; AIX = aortic augmentation index; BA = brachial artery; BMI = body mass index; CRP = C-reactive protein; DBP = diastolic blood pressure; FMD = flow-mediated dilation; Hb = hemoglobin; HDL = high density lipoprotein; HR = heart rate; LDL = low density lipoprotein; NMD = nitroglycerin mediated dilation; PU = perfusion units; PWV = pulse wave velocity; SBP = systolic blood pressure)

	Young	Elderly	<i>p-value</i>
<b>A</b>	20	19	
n			
Age (y)	26 ± 3	60 ± 8	<b>&lt;0.001</b>
BMI (kg/m <sup>2</sup> )	24.1 ± 2.3	26.5 ± 3.2	<b>0.011</b>
Height (m)	1.82 ± 0.06	1.82 ± 0.05	0.691
Weight (kg)	80 ± 10	87 ± 12	<b>0.044</b>
Creatinine (mg/dL)	1.0 ± 0.1	1.0 ± 0.1	0.729
Total cholesterol (mg/dL)	185 ± 35	223 ± 32	<b>0.001</b>
LDL cholesterol (mg/dL)	130 ± 26	152 ± 27	<b>0.029</b>
HDL cholesterol (mg/dL)	54 ± 17	54 ± 12	0.916
Triglycerides (mg/dL)	98 ± 46	119 ± 40	0.123
Fasting plasma glucose (mg/dL)	89 ± 8	94 ± 8	<b>0.045</b>
Hb <sub>A1c</sub> (%)	4.8 ± 1.2	4.5 ± 2.0	0.533
CRP (mg/dl)	0.1 ± 0.2	0.05 ± 0.1	0.214
Hb (mg/dl)	15.2 ± 0.9	15.4 ± 1.1	0.516
Leucocytes (1,000 /μL)	5.3 ± 1.2	5.7 ± 1.4	0.332
<b>B</b>			
BA resting diameter (mm)	4.40 ± 0.26	4.90 ± 0.48	<b>&lt;0.001</b>
FMD (%)	6.3 ± 0.6	5.1 ± 0.8	<b>&lt;0.001</b>
NMD (%)	14.9 ± 1.3	12.8 ± 1.7	<b>0.004</b>
HR (bpm)	56 ± 8	56 ± 7	0.780
Office SBP (mmHg)	121 ± 6	130 ± 11	<b>0.003</b>
Office DBP (mmHg)	77 ± 7	82 ± 9	0.073
Central SBP (mmHg)	104 ± 7	124 ± 14	<b>&lt;0.001</b>
Central DBP (mmHg)	77 ± 7	84 ± 9	<b>0.016</b>
PWV (m/s)	5.9 ± 0.5	9.3 ± 1.3	<b>&lt;0.001</b>
AIX (%)	-8 ± 9	23 ± 8	<b>&lt;0.001</b>
Microvascular perfusion baseline (PU)	41 ± 14	40 ± 8	0.798
Microvascular perfusion maximum (PU)	264 ± 52	184 ± 36	<b>&lt;0.001</b>

**Table 2:** Composition of *FLAVANOL* *CONTROL* interventional vehicles ingested bi-daily (ND=not detectable) as used in previous studies.<sup>8, 19, 35, 36</sup>

Total cocoa flavanols (mg)*	450	ND
Monomers (mg)**	73	ND
(-)-Epicatechin (mg)**	64	ND
(-)-Catechin (mg)**	7	ND
(+)-Catechin (mg)**	2	ND
(+)-Epicatechin (mg)**	ND	ND
Dimers-decamers (mg)*	377	ND
Theobromine (mg)***	44	46
Caffeine (mg)***	10	6
Fat (g) <sup>#</sup>	0	0
Carbohydrates (g) <sup>##</sup>	6	6
Protein (g) <sup>#</sup>	0.1	0.1
Energy (kcal) <sup>#</sup>	25	25
Sodium (mg) <sup>#</sup>	3	3
Potassium (mg) <sup>#</sup>	95	85

\*Content determined using method by Adamson et al.,<sup>17</sup> with a %RSD (percent relative standard deviation) of 6%; \*\*Content determined using method by Machonis et al.,<sup>37</sup> with a %RSD of 2%, 3%, and 10% for (–)-epicatechin, (–)-catechin and (+)-catechin, respectively; \*\*\*Typical %RSD of 3% are observed for both caffeine and theobromine; <sup>#</sup>Macronutrients were determined using AOAC-accredited methods used for nutritional product labelling. <sup>##</sup> Content determined using Total Carbohydrate by difference method.

**Table 3:** Univariate correlations between age, CD31<sup>+</sup>/41<sup>-</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup> endothelial microparticles (EMPs), clinical, and hemodynamic parameters. (r is Pearson's correlation coefficient; Ev/ $\mu$ L = events on flow cytometry analysis per microliter platelet free plasma; AIX = aortic augmentation index; BA = brachial artery; DBP = diastolic blood pressure; FMD = flow-mediated dilation; HDL = high density lipoprotein; LDL = low density lipoprotein; PU = perfusion units; PWV = pulse wave velocity; SBP = systolic blood pressure)

	Age (y)		CD31 <sup>+</sup> /41 <sup>-</sup> (Ev/ $\mu$ L)		CD144 <sup>+</sup> (Ev/ $\mu$ L)		CD62e <sup>+</sup> (Ev/ $\mu$ L)	
	r	p	r	p	r	p	r	p
<b>A</b> Age (y)			<b>0.37</b>	<b>0.019</b>	<b>0.38</b>	<b>0.018</b>	<b>0.53</b>	<b>0.001</b>
Fasting plasma glucose (mg/dL)	<b>0.39</b>	<b>0.013</b>	0.30	0.067	0.23	0.150	0.28	0.083
BMI (kg/m <sup>2</sup> )	<b>0.42</b>	<b>0.008</b>	<b>0.32</b>	<b>0.047</b>	0.23	0.163	<b>0.33</b>	<b>0.043</b>
Triglycerides (mg/dL)	0.24	0.149	<b>0.32</b>	<b>0.046</b>	0.07	0.657	0.20	0.226
Total cholesterol (mg/dL)	<b>0.55</b>	<b>&lt;0.001</b>	0.14	0.395	0.20	0.217	0.28	0.088
LDL cholesterol (mg/dL)	<b>0.41</b>	<b>0.021</b>	0.10	0.597	0.20	0.285	<b>0.42</b>	<b>0.017</b>
HDL cholesterol (mg/dL)	0.03	0.889	-0.28	0.121	-0.17	0.365	0.32	0.072
Leucocytes (1,000 / $\mu$ L)	0.22	0.184	<b>0.39</b>	<b>0.015</b>	0.08	0.644	<b>0.32</b>	<b>0.049</b>
<b>B</b> HR (/min)	0.07	0.698	0.01	0.940	-0.21	0.229	-0.15	0.395
BA resting diameter (mm)	<b>0.59</b>	<b>&lt;0.001</b>	0.21	0.209	0.05	0.722	<b>0.47</b>	<b>0.003</b>
FMD (%)	<b>-0.40</b>	<b>0.011</b>	<b>-0.39</b>	<b>0.013</b>	0.05	0.744	<b>-0.28</b>	<b>0.048</b>
Office SBP (mmHg)	<b>0.43</b>	<b>0.007</b>	<b>0.38</b>	<b>0.016</b>	<b>0.34</b>	<b>0.032</b>	<b>0.32</b>	<b>0.047</b>
Office DBP (mmHg)	<b>0.32</b>	<b>0.046</b>	0.19	0.236	0.21	0.195	0.17	0.292
Central SBP (mmHg)	<b>0.68</b>	<b>&lt;0.001</b>	<b>0.44</b>	<b>0.005</b>	<b>0.43</b>	<b>0.007</b>	<b>0.42</b>	<b>0.008</b>
Central DBP (mmHg)	<b>0.43</b>	<b>0.007</b>	0.10	0.571	0.24	0.146	0.3	0.061
PWV (m/s)	<b>0.82</b>	<b>&lt;0.001</b>	<b>0.42</b>	<b>0.008</b>	<b>0.35</b>	<b>0.031</b>	<b>0.41</b>	<b>0.010</b>
AIX (%)	<b>0.81</b>	<b>&lt;0.001</b>	<b>0.45</b>	<b>0.004</b>	0.28	0.087	<b>0.47</b>	<b>0.002</b>
Microvascular perfusion maximum (PU)	<b>-0.67</b>	<b>&lt;0.001</b>	<b>-0.41</b>	<b>0.011</b>	<b>-0.35</b>	<b>0.033</b>	<b>-0.46</b>	<b>0.004</b>

347 **Table 4:** Univariate correlations between changes in CD31<sup>+</sup>/41<sup>+</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup> endothelial microparticles (EMPs), clinical and hemodynamic parameters.  
348 (r is Pearson’s correlation coefficient; Ev/ $\mu$ L = events on flow cytometry analysis per microliter platelet free plasma; AIX = aortic augmentation index; BA =  
349 brachial artery; DBP = diastolic blood pressure; FMD = flow-mediated dilation; PU = perfusion units; PWV = pulse wave velocity; SBP = systolic blood  
350 pressure)  
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	CD31 <sup>+</sup> /41 <sup>+</sup> (Delta Ev/ $\mu$ L)		CD144 <sup>+</sup> (Delta Ev/ $\mu$ L)		CD62e <sup>+</sup> (Delta Ev/ $\mu$ L)	
	r	p	r	p	r	p
Total cholesterol (Delta mg/dL)	0.19	0.244	0.07	0.687	0.17	0.310
BA resting diameter (Delta mm)	0.19	0.253	-0.19	0.257	0.14	0.393
FMD (Delta %)	<b>-0.39</b>	<b>0.015</b>	<b>-0.51</b>	<b>0.001</b>	<b>-0.48</b>	<b>0.002</b>
Office SBP (Delta mmHg)	0.03	0.871	0.25	0.125	0.20	0.228
Office DBP (Delta mmHg)	0.29	0.069	0.23	0.151	0.23	0.155
Central SBP (Delta mmHg)	<b>0.34</b>	<b>0.035</b>	-0.01	0.962	<b>0.37</b>	<b>0.022</b>
Central DBP (Delta mmHg)	0.18	0.275	-0.01	0.975	0.19	0.243
PWV (Delta m/s)	0.12	0.466	<b>0.36</b>	<b>0.027</b>	<b>0.56</b>	<b>&lt;0.001</b>
AIX (Delta %)	0.30	0.064	<b>0.32</b>	<b>0.048</b>	0.19	0.245
Microvascular perfusion maximum (Delta PU)	-0.06	0.744	-0.21	0.196	0.01	0.953

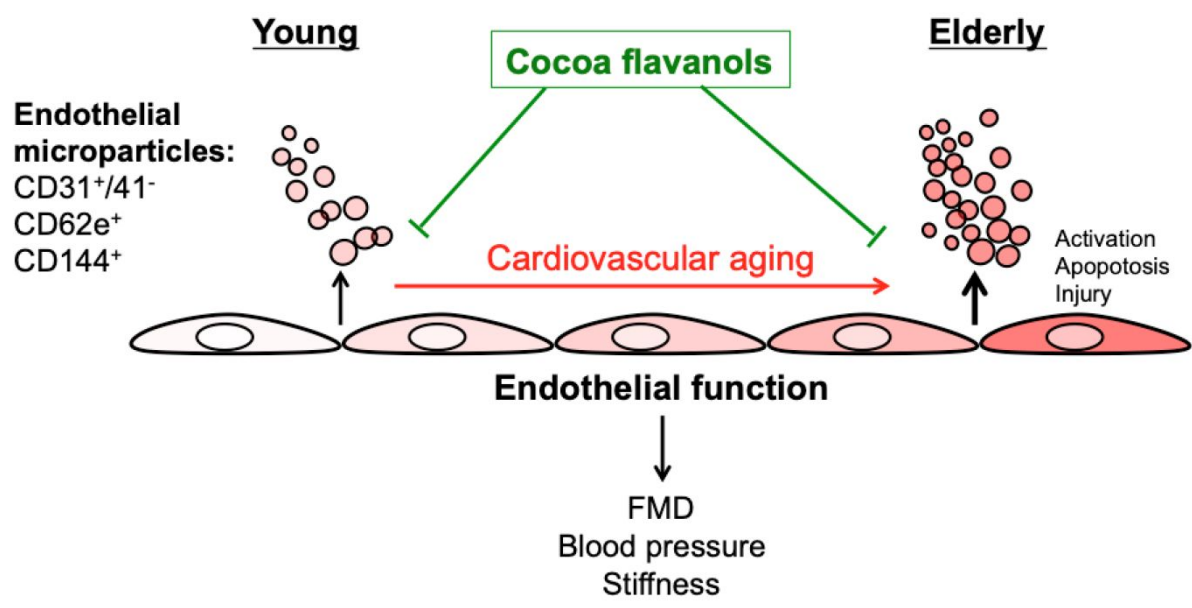
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**FIGURE LEGENDS****Graphic for table of contents**

**Figure 1: Concentrations of circulating endothelial microparticles (CD31<sup>+</sup>/41<sup>-</sup>, CD144<sup>+</sup>, CD62e<sup>+</sup>) are increased in elderly as compared to young subjects.** Values are mean +/- SEM of events on flow cytometry analysis per microliter platelet free plasma (Ev/ $\mu$ L), \* *p* vs *YOUNG* <0.05 (*t*-test).

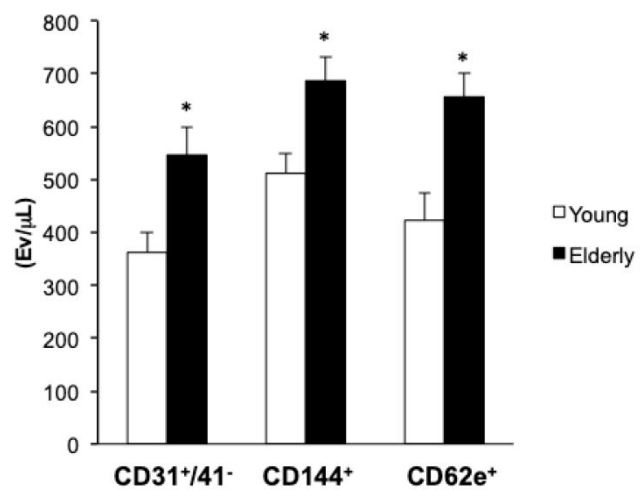
**Figure 2: Change in concentrations of circulating endothelial microparticles (CD31<sup>+</sup>/41<sup>-</sup>, CD144<sup>+</sup>, CD62e<sup>+</sup>) in young and elderly following 2 weeks of twice daily cocoa flavanol ingestion.** Values are mean change +/- SEM of events on flow cytometry analysis per microliter platelet free plasma (Ev/ $\mu$ L), \* *p* *FLAVANOL* vs *CONTROL* <0.05. (ANOVA; interaction between treatment and group were not statistically significant in all microparticle subgroups)





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366 **Graphic for table of contents**



**Figure 1**

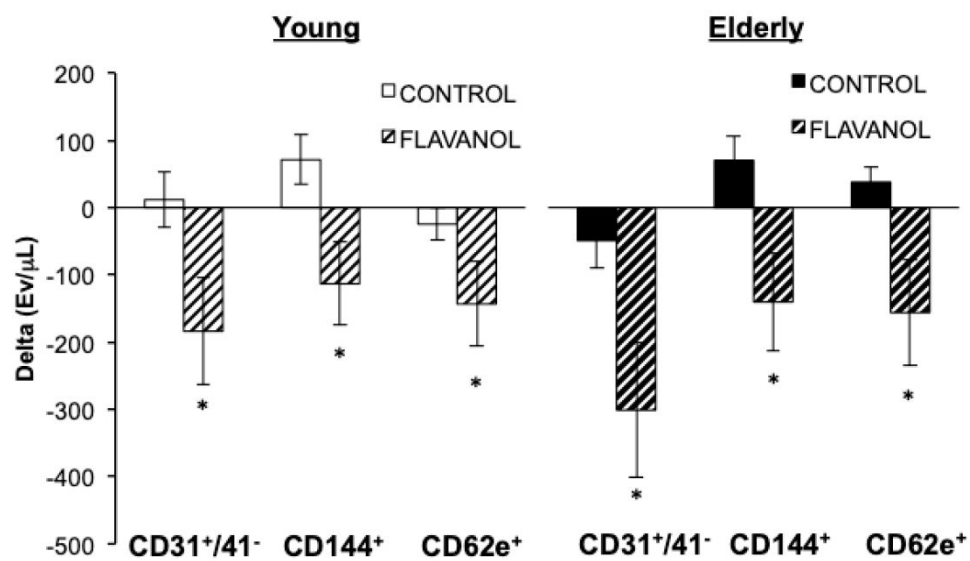


Figure 2